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(54) Title: DIAGNOSTIC MARKERS OF ACUTE CORONARY SYNDROMES AND METHODS OF USE THEREOF

(57) **Abstract:** The present invention relates to methods for the diagnosis and evaluation of acute coronary syndromes. In particular, patient test samples are analyzed for the presence and amount of members of a panel of markers comprising one or more specific markers for myocardial injury and one or more non-specific markers for myocardial injury. A variety of markers are disclosed for assembling a panel of markers for such diagnosis and evaluation. In various aspects, the invention provides methods for the early detection and differentiation of stable angina, unstable angina, and myocardial infarction. Invention methods provide rapid, sensitive and specific assays that can greatly increase the number of patients that can receive beneficial treatment and therapy, reduce the costs associated with incorrect diagnosis, and provide important information about the prognosis of the patient.

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DIAGNOSTIC MARKERS OF ACUTE CORONARY SYNDROMES AND METHODS OF USE THEREOF

[0001] This application is related to and claims priority from U.S. Provisional Patent Application No. 60/288,871, filed on May 4, 2001 (Atty Docket No. 071949-6501) ; and U.S. Provisional Patent Application No. 60/315,642, filed on August 28, 2001 (Atty Docket No. 071949-5501), each of which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to the identification and use of diagnostic markers for acute coronary syndromes (ACS). In various aspects, the invention relates to methods for the early detection and differentiation of ACS and the identification of individuals at risk for adverse events upon presentation with ACS symptoms.

BACKGROUND OF THE INVENTION

[0003] The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

[0004] ACS is a manifestation of vascular injury to the heart, also referred to as myocardial injury or myocardial damage, that is commonly secondary to atherosclerosis or hypertension, and is the leading cause of death in the United States. ACS is commonly caused by occlusion associated with coronary artery disease cause by atherosclerotic plaque formation and progression to either further occlusion or fissure. ACS can be manifested as stable angina, unstable angina, or myocardial infarction.

[0005] The term "acute coronary syndromes" ("ACS") has been applied to a group of coronary disorders that result from ischemic insult to the heart. Patients with ACS form a heterogeneous group, with differences in pathophysiology, clinical presentation, and risk for adverse events. Such patients present to the physician with conditions that span a continuum that includes unstable angina, non-ST-elevation non-Q wave myocardial infarction ("NST"-"MI"), ST-elevation non-Q wave MI, and transmural (Q-wave) MI. ACS is believed to result largely from thrombus deposition and growth

within one or more coronary arteries, resulting in a partial or complete occlusion of the artery, and frequently involves rupture of the plaque, resulting in an ischemic injury. ACS may also be precipitated by a coronary vasospasm or increased myocardial demand. For review, *see, e.g.*, Davies, *Clin. Cardiol.* **20** (Supp. I): I2-I7 (1997).

[0006] The seriousness of ACS is underlined by the morbidity and mortality that follow the ischemic insult. For example, workers have estimated that within four to six weeks of presentation with ACS, the risk of death or a subsequent myocardial infarction (MI) is 8-14%, and the rate of death, MI, or refractory ischemia is 15-25% (Theroux and Fuster, *Circulation* **97**: 1195-1206, 1998). Given that the total number of deaths in the U.S. from acute MI is about 600,000, the search within the art for information that relates to the diagnosis, prognosis, and management of ACS has understandably been extensive. Several potential markers that may provide such information in certain patient populations have been identified, including circulating cardiac troponin levels (*see, e.g.*, Antman et al., *N. Eng. J. Med.* **335**: 1342-9, 1996; see also U.S. Patent Nos. 6,147,688, 6,156,521, 5,947,124, and 5,795,725, each of which is hereby incorporated by reference in its entirety), ST-segment depression (*see, e.g.*, Savonitto et al., *JAMA* **281**: 707-13, 1999), circulating creatine kinase levels (*see, e.g.*, Alexander et al., *Circulation* (Suppl.) 1629, 1998), and circulating c-reactive protein levels (*see, e.g.*, Morrow et al., *J. Am. Coll. Cardiol.* **31**: 1460-5, 1998).

[0007] Stable angina is characterized by constricting chest pain that occurs upon exertion or stress, and is relieved by rest or sublingual nitroglycerin. Unstable angina is characterized by constricting chest pain at rest that is relieved by sublingual nitroglycerin. Anginal chest pain is usually relieved by sublingual nitroglycerin, and the pain usually subsides within 30 minutes. Myocardial infarction is characterized by constricting chest pain lasting longer than 30 minutes that can be accompanied by diagnostic electrocardiography (ECG) Q waves. Unstable angina is thought to represent the clinical state between stable angina and myocardial infarction, and is commonly associated with atherosclerotic plaque rupture and thrombus formation. In this regard, atherosclerotic plaque rupture is the most common cause of myocardial infarction.

[0008] Inflammation occurs during stable angina, and markers of plaque rupture, platelet activation, and early thrombosis can be used to identify and monitor the

progressing severity of unstable angina. The myocardial damage caused during an anginal attack is, by definition, reversible, while damage caused during a myocardial infarction is irreversible. According to this model, a specific marker of myocardial injury can be used to identify myocardial infarction. The progression of coronary artery disease from mild unstable angina to severe unstable angina and myocardial infarction is related to plaque instability and the degree of arterial occlusion. This progression can occur slowly, as stable plaques enlarge and become more occlusive, or it can occur rapidly, as unstable plaques rupture, causing platelet activation and occlusive thrombus formation. Because myocardial infarction most frequently shares the same pathophysiology as unstable angina, it is possible that the only distinction between these two events is the reversibility of myocardial damage. However, since the only distinction between severe unstable angina and mild myocardial infarction is based on clinical judgement, markers of myocardial damage may also appear in the peripheral circulation of patients diagnosed as having unstable angina.

[0009] Current diagnostic methods for ACS commonly include clinical symptoms, electrocardiography (ECG), and the measurement of cardiac markers in the peripheral circulation. Angiography is also used in cases of severe chest pain usually associated with unstable angina and acute myocardial infarction (AMI). Patients with ACS frequently have constricting chest pain that often radiates to the neck, jaw, shoulders, or down the inside of the left or both arms and can have accompanying symptoms of dyspnea, diaphoresis, palpitations, light-headedness, and nausea. Myocardial ischemia can produce diagnostic ECG changes including Q waves and ST segment changes. Elevations of the plasma concentration of cardiac enzymes may reflect the degree of cardiac tissue necrosis associated with severe unstable angina and myocardial infarction.

[0010] Accordingly, there is a present need in the art for a rapid, sensitive and specific diagnostic assay for ACS that can also differentiate the type of ACS and identify those individuals at risk for delayed adverse events. Such a diagnostic assay would greatly increase the number of patients that can receive beneficial treatment and therapy, and reduce the costs associated with incorrect diagnosis.

SUMMARY OF THE INVENTION

[0011] The present invention relates to the identification and use of diagnostic and/or prognostic markers for ACS, ischemia, and/or necrosis. The methods and compositions described herein can meet the need in the art for a rapid, sensitive and specific diagnostic assay to be used in the diagnosis, differentiation and prognosis of various forms of ACS. Moreover, the methods and compositions of the present invention can also be used to facilitate the treatment of ACS patients and the development of additional diagnostic indicators.

[0012] The terms "ischemia and ischemic" relate to damage to the myocardium as a result of a reduction of blood flow to the heart. The terms "angina pectoris", "stable angina", "unstable angina", "silent ischemia" are generally related to myocardial ischemia. One skilled in the art will recognize these terms, which are described in "The Merck Manual of Diagnosis and Therapy" Seventeenth Edition, 1999, Ed. Keryn A.G. Lane, pp. 1662-1668, incorporated by reference only. The term ischemia is also related to what one skilled in the art would consider as minor myocardial injury or damage. The term ischemia is further described in the Journal of the American College of Cardiology **36**, 959-969 (2000), incorporated by reference only.

[0013] The terms "necrosis and necrotic" relate to myocardial cell death as a result of a reduction or stoppage of blood flow to the heart. Myocardial necrosis is a condition of the heart which is more severe than myocardial ischemia. The term "myocardial infarction" is generally related to myocardial necrosis. One skilled in the art will recognize these terms, which are described in "The Merck Manual of Diagnosis and Therapy" Seventeenth Edition, 1999, Ed. Keryn A.G. Lane, pp. 1668-1677, incorporated by reference only. The term necrosis is also related to what one skilled in the art would consider as major myocardial injury or damage. The terms myocardial infarction and necrosis are further described in the Journal of the American College of Cardiology **36**, 959-969 (2000), incorporated by reference only.

[0014] In various aspects, the invention relates to materials and procedures for identifying markers that are associated with the diagnosis, prognosis, or differentiation of ACS in a patient; to using such markers in diagnosing and treating a patient and/or to monitor the course of a treatment regimen; and for screening compounds and

pharmaceutical compositions that might provide a benefit in treating or preventing such conditions.

[0015] In a first aspect, the invention features methods of diagnosing ACS by analyzing a test sample obtained from a patient for the presence or amount of one or more markers for myocardial injury. These methods can include identifying one or more markers, the presence or amount of which is associated with the diagnosis, prognosis, or differentiation of ACS. Once such a marker(s) is identified, the level of such a marker(s) in a patient sample can be measured. In certain embodiments, these markers can be compared to a diagnostic level that is associated with the diagnosis, prognosis, or differentiation of ACS. By correlating the patient level to the diagnostic level, the presence or absence of ACS, and the probability of future adverse outcomes in a patient may be rapidly and accurately determined.

[0016] For purposes of the following discussion, the methods described as applicable to the diagnosis and prognosis of myocardial infarction generally may be considered applicable to the diagnosis and prognosis of stable angina and unstable angina.

[0017] In certain embodiments, a plurality of markers are combined to increase the predictive value of the analysis in comparison to that obtained from the markers individually or in smaller groups. Preferably, one or more specific markers for myocardial injury can be combined with one or more non-specific markers for myocardial injury to enhance the predictive value of the described methods.

[0018] The term "marker" as used herein refers to molecules to be used as targets for screening patient test samples. Examples of such molecular targets are proteins or polypeptides. "Proteins or polypeptides" used as markers in the present invention are contemplated to include any fragments thereof, in particular, immunologically detectable fragments. One of skill in the art would recognize that proteins which are released by cells of the heart which become damaged during vascular injury could become degraded or cleaved into such fragments. Additionally, certain markers are synthesized in an inactive form, which may be subsequently activated by proteolysis. Examples of such markers are described hereinafter. The term "related marker" as used

herein refers to one or more fragments of a particular marker that may be detected as a surrogate for the marker itself.

[0019] To date, BNP and BNP related peptides have not been used as markers of myocardial ischemia. Additionally, other markers of various pathological processes including inflammation, coagulation, and plaque rupture have not been used as subsets of a larger panel of markers of myocardial ischemia. Preferred markers of the invention can aid in the diagnosis, differentiation, and prognosis of patients with myocardial infarction, unstable angina, and stable angina.

[0020] The term "test sample" as used herein refers to a biological sample obtained for the purpose of diagnosis, prognosis, or evaluation. In certain embodiments, such a sample may be obtained for the purpose of determining the outcome of an ongoing condition or the effect of a treatment regimen on a condition. Preferred test samples include blood, serum, plasma, cerebrospinal fluid, urine and saliva. In addition, one of skill in the art would realize that some test samples would be more readily analyzed following a fractionation or purification procedure, for example, separation of whole blood into serum or plasma components.

[0021] The term "specific marker of myocardial injury" as used herein refers to molecules that are typically associated with cardiac tissue, and which can be correlated with a cardiac injury, but are not correlated with other types of injury. Such specific markers of cardiac injury include annexin V, B-type natriuretic peptide, β-enolase, cardiac troponin I (free and/or complexed), cardiac troponin T (free and/or complexed), creatine kinase-MB, glycogen phosphorylase-BB, heart-type fatty acid binding protein, phosphoglyceric acid mutase-MB, and S-100ao. These specific markers are described in detail hereinafter.

[0022] The term "non-specific marker of myocardial injury" as used herein refers to molecules that are typically general markers of coagulation and hemostasis or acute phase reactants. Such markers may be elevated in the event of cardiac injury, but may also be elevated due to non-cardiac events. Factors in the activation of platelets and the mechanisms of coagulation include β-thromboglobulin, D-dimer, fibrinopeptide A, platelet-derived growth factor, plasmin-α-2-antiplasmin complex, platelet factor 4, prothrombin fragment 1+2, P-selectin, thrombin-antithrombin III complex, thrombus

precursor protein, tissue factor, and von Willebrand factor. These non-specific markers are described in detail hereinafter.

[0023] The term "acute phase reactants" as used herein refers to proteins whose concentrations are elevated in response to stressful or inflammatory states that occur during various insults that include infection, injury, surgery, trauma, tissue necrosis, and the like. Acute phase reactant expression and serum concentration elevations are not specific for the type of insult, but rather as a part of the homeostatic response to the insult.

[0024] All acute phase reactants are produced in response to insult, perhaps in order to handle extensive insult, even though some components may not be needed. Examples of classical acute phase proteins include C-reactive protein, ceruloplasmin, fibrinogen, α1-acid glycoprotein, α1-antitrypsin, and haptoglobin. Various cytokines and related molecules such as insulin-like growth factor-1, interleukin-1β, interleukin-1 receptor antagonist, interleukin-6, interleukin-8, transforming growth factor β, monocyte chemotactic protein-1, and tumor necrosis factor α are components of the inflammatory response that are also intimately involved in the acute phase reaction. Such cytokines are released into the bloodstream from the site of insult and are capable of themselves inducing expression of other acute phase proteins.

[0025] Other non-specific markers of myocardial injury include markers of atheroslcerotic plaque rupture. An atheroscloerotic plaque consists of accumulated lipids, smooth muscle cells, connective tissue, and glycosaminoglycans. Vessels containing such plaques have reduced systolic expansion, abnormally rapid wave propagation, and progressively reduced elasticity as plaque formation progresses. A plaque may progress to severe stenosis and total arterial occlusion. Some plaques are stable, but others which are rich in lipids and inflammatory cells typically have a thin fibrous cap and may undergo spontaneous rupture. These unstable plaques are more closely associated with the onset of an acute ischemic event. Therefore, markers of atherosclerotic plaque rupture may be useful in the diagnosis and evaluation of potential ACS victims. Such markers of atherosclerotic plaque rupture inclued human neutrophil elastase, inducible nitric oxide synthase, lysophosphatidic acid, malondialdehyde-modified low-density lipoprotein, matrix metalloproteinase-1, matrix metalloproteinase-2, matrix metalloproteinase-3, and matrix metalloproteinase-9.

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[0026] Other non-specific markers of myocardial injury may include caspase-3, hemoglobin α_2, soluble intercellular adhesion molecule-1 and soluble vascular cell adhesion molecule-1.

[0027] The phrase "diagnosis" as used herein refers to methods by which the skilled artisan can estimate and even determine whether or not a patient is suffering from a given disease or condition. The skilled artisan often makes a diagnosis on the basis of one or more diagnostic indicators, i.e., a marker, the presence, absence, or amount of which is indicative of the presence, severity, or absence of the condition.

[0028] Similarly, a prognosis is often determined by examining one or more "prognostic indicators." These are markers, the presence or amount of which in a patient (or a sample obtained from the patient) signal a probability that a given course or outcome will occur. For example, when one or more prognostic indicators reach a sufficiently high level in samples obtained from such patients, the level may signal that the patient is at an increased probability for experiencing a future event in comparison to a similar patient exhibiting a lower marker level. A level or a change in level of a prognostic indicator, which in turn is associated with an increased probability of morbidity or death, is referred to as being "associated with an increased predisposition to an adverse outcome" in a patient. Preferred prognostic markers can predict the onset of delayed adverse events in a patient, or the chance of future ACS.

[0029] The term "correlating," as used herein in reference to the use of diagnostic and prognostic indicators, refers to comparing the presence or amount of the indicator in a patient to its presence or amount in persons known to suffer from, or known to be at risk of, a given condition; or in persons known to be free of a given condition, i.e. "normal individuals". For example, a marker level in a patient sample can be compared to a level known to be associated with a specific type of ACS. The sample's marker level is said to have been correlated with a diagnosis; that is, the skilled artisan can use the marker level to determine whether the patient suffers from a specific type of ACS, and respond accordingly. Alternatively, the sample's marker level can be compared to a marker level known to be associated with a good outcome (e.g., the absence of ACS), such as an average level found in a population of normal individuals.

[0030] In certain embodiments, a diagnostic or prognostic indicator is correlated to a condition or disease by merely its presence or absence. In other embodiments, a threshold level of a diagnostic or prognostic indicator can be established, and the level of the indicator in a patient sample can simply be compared to the threshold level. A preferred threshold level for markers of the present invention is about 25 pg/mL, about 50 pg/mL, about 60 pg/mL, about 75 pg/mL, about 100 pg/mL, about 150 pg/mL, about 200 pg/mL, about 300 pg/mL, about 400 pg/mL, about 500 pg/mL, about 600 pg/mL, about 750 pg/mL, about 1000 pg/mL, and about 2500 pg/mL. The term "about" in this context refers to +/- 10%.

[0031] In yet other embodiments, multiple determination of one or more diagnostic or prognostic markers can be made, and a temporal change in the marker can be used to determine a diagnosis or prognosis. For example, a diagnostic indicator may be determined at an initial time, and again at a second time. In such embodiments, an increase in the marker from the initial time to the second time may be diagnostic of a particular type of ACS, or a given prognosis. Likewise, a decrease in the marker from the initial time to the second time may be indicative of a particular type of ACS, or a given prognosis. Furthermore, the degree of change of one or more markers may be related to the severity of ACS and future adverse events.

[0032] In yet another embodiment, multiple determination of one or more diagnostic or prognostic markers can be made, and a temporal change in the marker can be used to monitor the efficacy of appropriate therapies. In such an embodiment, one might expect to see a decrease or an increase in the marker(s) over time during the course of effective therapy.

[0033] The skilled artisan will understand that, while in certain embodiments comparative measurements are made of the same diagnostic marker at multiple time points, one could also measure a given marker at one time point, and a second marker at a second time point, and a comparison of these markers may provide diagnostic information.

[0034] The phrase "determining the prognosis" as used herein refers to methods by which the skilled artisan can predict the course or outcome of a condition in a patient. The term "prognosis" does not refer to the ability to predict the course or outcome of a

condition with 100% accuracy, or even that a given course or outcome is predictably more or less likely to occur based on the presence, absence or levels of test markers. Instead, the skilled artisan will understand that the term "prognosis" refers to an increased probability that a certain course or outcome will occur; that is, that a course or outcome is more likely to occur in a patient exhibiting a given condition, when compared to those individuals not exhibiting the condition. For example, in individuals not exhibiting the condition, the chance of a given outcome may be about 3%. In preferred embodiments, a prognosis is about a 5% chance of a given outcome, about a 7% chance, about a 10% chance, about a 12% chance, about a 15% chance, about a 20% chance, about a 25% chance, about a 30% chance, about a 40% chance, about a 50% chance, about a 60% chance, about a 75% chance, about a 90% chance, and about a 95% chance. The term "about" in this context refers to +/- 1%.

[0035] The skilled artisan will understand that associating a prognostic indicator with a predisposition to an adverse outcome is a statistical analysis. For example, a marker level of greater than 80 pg/mL may signal that a patient is more likely to suffer from an adverse outcome than patients with a level less than or equal to 80 pg/mL, as determined by a level of statistical significance. Additionally, a change in marker concentration from baseline levels may be reflective of patient prognosis, and the degree of change in marker level may be related to the severity of adverse events. Statistical significance is often determined by comparing two or more populations, and determining a confidence interval and/or a p value. See, e.g., Dowdy and Wearden, Statistics for Research, John Wiley & Sons, New York, 1983. Preferred confidence intervals of the invention are 90%, 95%, 97.5%, 98%, 99%, 99.5%, 99.9% and 99.99%, while preferred p values are 0.1, 0.05, 0.025, 0.02, 0.01, 0.005, 0.001, and 0.0001. Exemplary statistical tests for associating a prognostic indicator with a predisposition to an adverse outcome are described hereinafter.

[0036] In other embodiments, a threshold degree of change in the level of a prognostic or diagnostic indicator can be established, and the degree of change in the level of the indicator in a patient sample can simply be compared to the threshold degree of change in the level. A preferred threshold change in the level for markers of the invention is about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 50%, about 75%, about 100%, and about 150%. The term "about" in this context

refers to +/- 10%. In yet other embodiments, a "nomogram" can be established, by which a level of a prognostic or diagnostic indicator can be directly related to an associated disposition towards a given outcome. The skilled artisan is acquainted with the use of such nomograms to relate two numeric values with the understanding that the uncertainty in this measurement is the same as the uncertainty in the marker concentration because individual sample measurements are referenced, not population averages.

[0037] In yet another aspect, the invention relates to methods for determining a treatment regimen for use in a patient diagnosed with ACS. The methods preferably comprise determining a level of one or more diagnostic or prognostic markers as described herein, and using the markers to determine a diagnosis for a patient. One or more treatment regimens that improve the patient's prognosis by reducing the increased disposition for an adverse outcome associated with the diagnosis can then be used to treat the patient. Such methods may also be used to screen pharmacological compounds for agents capable of improving the patients prognosis as above.

[0038] In a further aspect, the invention relates to kits for determining the diagnosis or prognosis of a patient. These kits preferably comprise devices and reagents for measuring one or more marker levels in a patient sample, and instructions for performing the assay. Optionally, the kits may contain one or more means for converting marker level(s) to a prognosis. Such kits preferably contain sufficient reagents to perform one or more such determinations.

DETAILED DESCRIPTION OF THE INVENTION

[0039] In accordance with the present invention, there are provided methods and compositions for the identification and use of markers that are associated with the diagnosis, prognosis, or differentiation of ACS in a patient. Such markers can be used in diagnosing and treating a patient and/or to monitor the course of a treatment regimen; and for screening compounds and pharmaceutical compositions that might provide a benefit in treating or preventing such conditions.

[0040] Myocardial ischemia is caused by an imbalance of myocardial oxygen supply and demand. Specifically, demand exceeds supply due to inadequate blood supply. The heart accounts for a small percentage of total body weight, but is

responsible for 7% of body oxygen consumption. Cardiac tissue metabolism is highly aerobic and has very little reserve to compensate for inadequate blood supply. When the blood supply is reduced to levels that are inadequate for myocardial demand, the tissue rapidly becomes hypoxic and toxic cellular metabolites can not be removed. Myocardial cells rapidly use oxygen supplies remaining in the local microvasculature, and the length of time that aerobic metabolism continues is indirectly proportional to the degree of arterial occlusion. Once the oxygen supply has been exhausted, oxidative phosphorylation can not continue because oxygen is no longer available as an electron acceptor, pyruvate can not be converted to acetyl coenzyme A and enter the citric acid cycle. Myocardial metabolism switches to anaerobic metabolism using glycogen and glucose stores, and pyruvate is fermented to lactate. Lactate accumulation is the primary cause of chest pain in individuals with ACS. As ischemia continues, cardiac tissue becomes more acidic as lactate and other acidic intermediates accumulate, ATP levels decrease, and available energy sources are depleted. Cardiac tissue can recover if it is reperfused 15-20 minutes after an ischemic event. After the cellular glycogen stores have been depleted, the cell gradually displays features of necrosis, including mitochondrial swelling and loss of cell membrane integrity. Upon reperfusion, these damaged cells die, possibly as a result of the cell's inability to maintain ionic equilibrium. A loss of membrane integrity causes the cell's cytosolic contents to be released into the circulation.

[0041] Stable angina, unstable angina, and myocardial infarction all share one common feature: constricting chest pain associated with myocardial ischemia. Angina is classified as stable or unstable through a physician's interpretation of clinical symptoms, with or without diagnostic ECG changes. The classification of angina as "stable" or "unstable" does not refer to the stability of the plaque itself, but rather, the degree of exertion that is required to elicit chest pain. Most notably, the classification of chest pain as stable or unstable angina (or even mild myocardial infarction) in cases other than definitive myocardial infarction is completely subjective. The diagnosis, and in this case the distinction, is made not by angiography, which may quantify the degree of arterial occlusion, but rather by a physician's interpretation of clinical symptoms.

[0042] Stable angina is characterized by constricting chest pain that occurs upon exertion or stress, and is relieved by rest or sublingual nitroglycerin. Coronary

angiography of patients with stable angina usually reveals 50-70% obstruction of at least one coronary artery. Stable angina is usually diagnosed by the evaluation of clinical symptoms and ECG changes. Patients with stable angina may have transient ST segment abnormalities, but the sensitivity and specificity of these changes associated with stable angina are low.

[0043] Unstable angina is characterized by constricting chest pain at rest that is relieved by sublingual nitroglycerin. Anginal chest pain is usually relieved by sublingual nitroglycerin, and the pain usually subsides within 30 minutes. There are three classes of unstable angina severity: class I, characterized as new onset, severe, or accelerated angina; class II, subacute angina at rest characterized by increasing severity, duration, or requirement for nitroglycerin; and class III, characterized as acute angina at rest. Unstable angina represents the clinical state between stable angina and AMI and is thought to be primarily due to the progression in the severity and extent of atherosclerosis, coronary artery spasm, or hemorrhage into non-occluding plaques with subsequent thrombotic occlusion. Coronary angiography of patients with unstable angina usually reveals 90% or greater obstruction of at least one coronary artery, resulting in an inability of oxygen supply to meet even baseline myocardial oxygen demand. Slow growth of stable atherosclerotic plaques or rupture of unstable atherosclerotic plaques with subsequent thrombus formation can cause unstable angina. Both of these causes result in critical narrowing of the coronary artery. Unstable angina is usually associated with atherosclerotic plaque rupture, platelet activation, and thrombus formation. Unstable angina is usually diagnosed by clinical symptoms, ECG changes, and changes in cardiac markers (if any). Treatments for patients with unstable angina include nitrates, aspirin, GPIIb/IIIa inhibitors, heparin, and beta-blockers. Thrombolytic therapy has not been demonstrated to be beneficial for unstable angina patients, and calcium channel blockers may have no effect. Patients may also receive angioplasty and stents. Finally, patients with unstable angina are at risk for developing AMI.

[0044] Myocardial infarction is characterized by constricting chest pain lasting longer than 30 minutes that can be accompanied by diagnostic ECG Q waves. Most patients with AMI have coronary artery disease, and as many as 25% of AMI cases are "silent" or asymptomatic infarctions, and individuals with diabetes tend to be more

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susceptible to silent infarctions. Population studies suggest that 20-60% of nonfatal myocardial infarctions are silent infarctions that are not recognized by the patient. Atypical clinical presentations of AMI can include congestive heart failure, angina pectoris without a severe or prolonged attack, atypical location of pain, central nervous system manifestations resembling stroke, apprehension and nervousness, sudden mania or psychosis, syncope, weakness, acute indigestion, and peripheral embolization. AMI is usually diagnosed by clinical symptoms, ECG changes, and elevations of cardiac proteins, most notably cardiac troponin, creatine kinase-MB and myoglobin. Treatments of AMI have improved over the past decade, resulting in improved patient outcome and a 30% decrease in the death rate associated with AMI. Treatment of AMI patients is accomplished by administering agents that limit infarct size and improve outcome by removing occlusive material, increasing the oxygen supply to cardiac tissue, or decreasing the oxygen demand of cardiac tissue. Treatments can include the following: supplemental oxygen, aspirin, GPIIb/IIIa inhibitors, heparin, thrombolytics (tPA), nitrates (nitroglycerin), magnesium, calcium channel antagonists, β-adrenergic receptor blockers, angiotensin-converting enzyme inhibitors, angioplasty (PTCA), and intraluminal coronary artery stents.

[0045] The 30 minute time point from chest pain onset is thought to represent the window of reversible myocardial damage caused by ischemia. Stable angina and unstable angina are characterized angiographically as 50-70% and 90% or greater arterial occlusion, respectively, and myocardial infarction is characterized by complete or nearly complete occlusion. A common misconception is that stable angina and unstable angina refer to plaque stability, or that they, along with myocardial infarction, are separate diseases. Because stable angina often progresses to unstable angina, and unstable angina often progresses to myocardial infarction, stable angina, unstable angina, and myocardial infarction can all be characterized as coronary artery disease of varying severity. Recently, the following physiological model of coronary artery disease progression has been proposed: Inflammation → Plaque Rupture → Platelet Activation → Early Thrombosis → Early Necrosis. This model is designed to fit the theory that inflammation occurs during stable angina, and that markers of plaque rupture, platelet activation, and early thrombosis can be used to identify and monitor the progressing severity of unstable angina. The myocardial damage caused during an anginal attack is, by definition, reversible, while damage caused during a myocardial

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infarction is irreversible. Therefore, there are two proposed break points in this model for the discrimination of stable angina, unstable angina, and AMI. The first occurs between inflammation and plaque rupture, with the theory that plaque rupture does not occur in stable angina. The second occurs between early thrombosis and early necrosis, with the theory that myocardial damage incurred during unstable angina is reversible. It is important to realize that these events, with the exception of early myocardial necrosis, can be associated with all forms of coronary artery disease, and that progression along this diagnostic pathway does not necessarily indicate disease progression. The progression of coronary artery disease from mild unstable angina to severe unstable angina and myocardial infarction is related to plaque instability and the degree of arterial occlusion. This progression can occur slowly, as stable plaques enlarge and become more occlusive, or it can occur rapidly, as unstable plaques rupture, causing platelet activation and occlusive thrombus formation. Because myocardial infarction most frequently shares the same pathophysiology as unstable angina, it is possible that the only distinction between these two events is the reversibility of myocardial damage. By definition, unstable angina causes reversible damage, while myocardial infarction causes irreversible damage. There have been published reports that indicate the presence of myocardial necrosis in patients with unstable angina. By definition, these patients may actually be experiencing early AMI. Nevertheless, even if these patients are diagnosed with unstable angina instead of early AMI, the high degree of severity suggests that they will benefit greatly from early aggressive treatment. Myocardial ischemia is the major determinant in the pathogenesis of stable angina, unstable angina, and myocardial infarction, and they should not be thought of as individual diseases. Rather, they reflect the increasing severity of myocardial damage from ischemia.

The Coagulation Cascade in ACS

[0046] There are essentially two mechanisms that are used to halt or prevent blood loss following vessel injury. The first mechanism involves the activation of platelets to facilitate adherence to the site of vessel injury. The activated platelets then aggregate to form a platelet plug that reduces or temporarily stops blood loss. The processes of platelet aggregation, plug formation and tissue repair are all accelerated and enhanced by numerous factors secreted by activated platelets. Platelet aggregation and plug

formation is mediated by the formation of a fibrinogen bridge between activated platelets. Concurrent activation of the second mechanism, the coagulation cascade, results in the generation of fibrin from fibrinogen and the formation of an insoluble fibrin clot that strengthens the platelet plug.

[0047] The coagulation cascade is an enzymatic pathway that involves numerous serine proteinases normally present in an inactive, or zymogen, form. The presence of a foreign surface in the vasculature or vascular injury results in the activation of the intrinsic and extrinsic coagulation pathways, respectively. A final common pathway is then followed, which results in the generation of fibrin by the serine proteinase thrombin and, ultimately, a crosslinked fibrin clot. In the coagulation cascade, one active enzyme is formed initially, which can activate other enzymes that active others, and this process, if left unregulated, can continue until all coagulation enzymes are activated. Fortunately, there are mechanisms in place, including fibrinolysis and the action of endogenous proteinase inhibitors that can regulate the activity of the coagulation pathway and clot formation.

[0048] Fibrinolysis is the process of proteolytic clot dissolution. In a manner analogous to coagulation, fibrinolysis is mediated by serine proteinases that are activated from their zymogen form. The serine proteinase plasmin is responsible for the degradation of fibrin into smaller degradation products that are liberated from the clot, resulting in clot dissolution. Fibrinolysis is activated soon after coagulation in order to regulate clot formation. Endogenous serine proteinase inhibitors also function as regulators of fibrinolysis.

[0049] Platelets are round or oval disks with an average diameter of 2-4 μm that are normally found in blood at a concentration of 200,000-300,000/μl. They play an essential role in maintaining hemostasis by maintaining vascular integrity, initially stopping bleeding by forming a platelet plug at the site of vascular injury, and by contributing to the process of fibrin formation to stabilize the platelet plug. When vascular injury occurs, platelets adhere to the site of injury and each other and are stimulated to aggregate by various agents released from adherent platelets and injured endothelial cells. This is followed by the release reaction, in which platelets secrete the contents of their intracellular granules, and formation of the platelet plug. The formation of fibrin by thrombin in the coagulation cascade allows for consolidation of

the plug, followed by clot retraction and stabilization of the plug by crosslinked fibrin. Active thrombin, generated in the concurrent coagulation cascade, also has the ability to induce platelet activation and aggregation.

[0050] The coagulation cascade can be activated through either the extrinsic or intrinsic pathways. These enzymatic pathways share one final common pathway. The result of coagulation activation is the formation of a crosslinked fibrin clot. Fibrinolysis is the process of proteolytic clot dissolution that is activated soon after coagulation activation, perhaps in an effort to control the rate and amount of clot formation. Urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) proteolytically cleave plasminogen, generating the active serine proteinase plasmin. Plasmin proteolytically digests crosslinked fibrin, resulting in clot dissolution and the production and release of fibrin degradation products.

[0051] The first step of the common pathway of the coagulation cascade involves the proteolytic cleavage of prothrombin by the factor Xa/factor Va prothrombinase complex to yield active thrombin. Thrombin is a serine proteinase that proteolytically cleaves fibrinogen to form fibrin, which is ultimately integrated into a crosslinked network during clot formation.

Exemplary Markers

(i) Specific Markers for Myocardial Injury

[0052] Annexin V, also called lipocortin V, endonexin II, calphobindin I, calcium binding protein 33, placental anticoagulant protein I, thromboplastin inhibitor, vascular anticoagulant-α, and anchorin CII, is a 33 kDa calcium-binding protein that is an indirect inhibitor and regulator of tissue factor. Annexin V is composed of four homologous repeats with a consensus sequence common to all annexin family members, binds calcium and phosphatidyl serine, and is expressed in a wide variety of tissues, including heart, skeletal muscle, liver, and endothelial cells (Giambanco, I. et al., *J. Histochem. Cytochem.* **39**:P1189-1198, 1991; Doubell, A.F. et al., *Cardiovasc. Res.* **27**:1359-1367, 1993). The normal plasma concentration of annexin V is < 2 ng/ml (Kaneko, N. et al., *Clin. Chim. Acta* **251**:65-80, 1996). The plasma concentration of annexin V is elevated in individuals with AMI (Kaneko, N. et al., *Clin. Chim. Acta* **251**:65-80, 1996). Due to its wide tissue distribution, elevation of the plasma

concentration of annexin V may be associated with any condition involving non-cardiac tissue injury. However, one study has found that plasma annexin V concentrations were not significantly elevated in patients with old myocardial infarction, chest pain syndrome, valvular heart disease, lung disease, and kidney disease (Kaneko, N. et al., *Clin. Chim. Acta* **251**:65-80, 1996). These previous results require confirmation before the clinical utility of annexin V as an ACS marker can be determined. Annexin V is released into the bloodstream soon after AMI onset. The annexin V concentration in the plasma of AMI patients decreased from initial (admission) values, suggesting that it is rapidly cleared from the bloodstream (Kaneko, N. et al., *Clin. Chim. Acta* **251**:65-80, 1996).

[0053] B-type natriuretic peptide (BNP), also called brain-type natriuretic peptide is a 32 amino acid, 4 kDa peptide that is involved in the natriuresis system to regulate blood pressure and fluid balance (Bonow, R.O., *Circulation* **93**:1946-1950, 1996). The precursor to BNP is synthesized as a 108-amino acid molecule, referred to as "pre pro BNP," that is proteolytically processed into a 76-amino acid N-terminal peptide (amino acids 1-76), referred to as "NT pro BNP" and the 32-amino acid mature hormone, referred to as BNP or BNP 32 (amino acids 77-108). It has been suggested that each of these species -- NT pro-BNP, BNP-32, and the pre pro BNP -- can circulate in human plasma (Tateyama et al., *Biochem. Biophys. Res. Commun.* **185**: 760-7 (1992); Hunt et al., *Biochem. Biophys. Res. Commun.* **214**: 1175-83 (1995)). The 2 forms, pre pro BNP and NT pro BNP, and peptides which are derived from BNP, pre pro BNP and NT pro BNP and which are present in the blood as a result of proteolyses of BNP, NT pro BNP and pre pro BNP, are collectively described as markers related to or associated with BNP. Proteolytic degradation of BNP and of peptides related to BNP have also been described in the literature and these proteolytic fragments are also encompassed it the term "BNP related peptides". BNP and BNP-related peptides are predominantly found in the secretory granules of the cardiac ventricles, and are released from the heart in response to both ventricular volume expansion and pressure overload (Wilkins, M. et al., *Lancet* **349**:1307-1310, 1997). Elevations of BNP are associated with raised atrial and pulmonary wedge pressures, reduced ventricular systolic and diastolic function, left ventricular hypertrophy, and myocardial infarction (Sagnella, G.A., *Clinical Science* **95**:519-529, 1998). Furthermore, there are numerous reports of elevated BNP concentration associated with congestive heart failure and

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renal failure. While BNP and BNP-related peptides are likely not specific for ACS, they may be sensitive markers of ACS because they may indicate not only cellular damage due to ischemia, but also a perturbation of the natriuretic system associated with ACS. The term "BNP" as used herein refers to the mature 32-amino acid BNP molecule itself. As the skilled artisan will recognize, however, other markers related to BNP may also serve as diagnostic or prognostic indicators in patients with ACS. For example, BNP is synthesized as a 108-amino acid pre pro-BNP molecule that is proteolytically processed into a 76-amino acid "NT pro BNP" and the 32-amino acid BNP molecule. Because of its relationship to BNP, the concentration of NT pro-BNP molecule can also provide diagnostic or prognostic information in patients. The phrase "marker related to BNP or BNP related peptide" refers to any polypeptide that originates from the pre pro-BNP molecule, other than the 32-amino acid BNP molecule itself. Thus, a marker related to or associated with BNP includes the NT pro-BNP molecule, the pro domain, a fragment of BNP that is smaller than the entire 32-amino acid sequence, a fragment of pre pro-BNP other than BNP, and a fragment of the pro domain. One skilled in the art will also recognize that the circulation contains proteases which can proteolyze BNP and BNP related molecules and that these proteolyzed molecules (peptides) are also considered to be "BNP related" and are additionally subjects of this invention.

[0054] Enolase is a 78 kDa homo- or heterodimeric cytosolic protein produced from α, β, and γ subunits. Enolase catalyzes the interconversion of 2-phosphoglycerate and phosphoenolpyruvate in the glycolytic pathway. Enolase is present as αα, αβ, ββ, αγ, and γγ isoforms. The α subunit is found in most tissues, the β subunit is found in cardiac and skeletal muscle, and the γ subunit is found primarily in neuronal and neuroendocrine tissues. β-enolase is composed of αβ and ββ enolase, and is specific for muscle. The normal plasma concentration of β-enolase is < 10 ng/ml (120 pM). β-enolase is elevated in the serum of individuals with AMI, but not in individuals with angina (Nomura, M. et al., *Br. Heart J.* **58**:29-33, 1987; Herraez-Domínguez, M.V. et al., *Clin. Chim. Acta* **64**:307-315, 1975). Further investigations into possible changes in plasma β-enolase concentration associated with unstable and stable angina need to be performed. The plasma concentration of β-enolase is elevated during heart surgery, muscular dystrophy, and skeletal muscle injury (Usui, A. et al., *Cardiovasc. Res.* **23**:737-740, 1989; Kato, K. et al., *Clin. Chim. Acta* **131**:75-85, 1983; Matsuda, H. et

al., *Forensic Sci. Int.* **99**:197-208, 1999). β-enolase is released into the bloodstream immediately following cardiac or skeletal muscle injury. The plasma β-enolase concentration was elevated to more than 150 ng/ml in the perioperative stage of cardiac surgery, and remained elevated for 1 week. Serum β-enolase concentrations peaked approximately 12-14 hours after the onset of chest pain and AMI and approached baseline after 1 week had elapsed from onset, with maximum levels approaching 1 μg/ml (Kato, K. et al., *Clin. Chim. Acta* **131**:75-85, 1983; Nomura, M. et al., *Br. Heart J.* **58**:29-33, 1987).

[0055] Troponin I (TnI) is a 25 kDa inhibitory element of the troponin complex, found in all striated muscle tissue. TnI binds to actin in the absence of Ca^{2+}, inhibiting the ATPase activity of actomyosin. A TnI isoform that is found in cardiac tissue (cTnI) is 40% divergent from skeletal muscle TnI, allowing both isoforms to be immunologically distinguished. The normal plasma concentration of cTnI is < 0.1 ng/ml (4 pM). The plasma cTnI concentration is elevated in patients with AMI. Investigations into changes in the plasma cTnI concentration in patients with unstable angina have yielded mixed results, but cTnI is not elevated in the plasma of individuals with stable angina (Benamer, H. et al., *Am. J. Cardiol.* **82**:845-850, 1998; Bertinchant, J.P. et al., *Clin. Biochem.* **29**:587-594, 1996; Tanasijevic, M.J. et al., *Clin. Cardiol.* **22**:13-16, 1999; Musso, P. et al., *J. Ital. Cardiol.* **26**:1013-1023, 1996; Holvoet, P. et al., *JAMA* **281**:1718-1721, 1999; Holvoet, P. et al., *Circulation* **98**:1487-1494, 1998). The mixed results associated with unstable angina suggest that cTnI may be useful in determining the severity of unstable angina because the extent of myocardial ischemia is directly proportional to unstable angina severity. The plasma cTnI concentration may be elevated in conjunction with cardiac trauma, congestive heart failure, and cardiac surgery, non-ischemic dilated cardiomyopathy, muscular disorders, CNS disorders, HIV infection, chronic renal failure, sepsis, lung disease, and endocrine disorders (Khan, I.A. et al., *Am. J. Emerg. Med.* **17**:225-229, 1999). This apparent non-specificity may be related to the quality and specificity of the antibodies used in the immunoassay. cTnI is released into the bloodstream following cardiac cell death. The plasma concentration of cTnI in patients with AMI is significantly elevated 4-6 hours after onset, peaks between 12-16 hours, and can remain elevated for one week. The release kinetics of cTnI associated with unstable angina may be similar. The measurement of specific forms of cardiac troponin, including free cardiac troponin I

and complexes of cardiac troponin I with troponin C and/or T may provide the user with the ability to identify various stages of ACS.

[0056] Free and complexed cardiac-troponin T may be used in a manner analogous to that described above for cardiac troponin I. Cardiac troponin T complex may be useful either alone or when expressed as a ratio with total cardiac troponin I to provide information related to the presence of progressing myocardial damage. Ongoing ischemia may result in the release of the cardiac troponin TIC complex, indicating that higher ratios of cardiac troponin TIC:total cardiac troponin I may be indicative of continual damage caused by unresolved ischemia.

[0057] Creatine kinase (CK) is a 85 kDa cytosolic enzyme that catalyzes the reversible formation ADP and phosphocreatine from ATP and creatine. CK is a homo- or heterodimer composed of M and B chains. CK-MB is the isoform that is most specific for cardiac tissue, but it is also present in skeletal muscle and other tissues. The normal plasma concentration of CK-MB is < 5 ng/ml. The plasma CK-MB concentration is significantly elevated in patients with AMI. Plasma CK-MB is not elevated in patients with stable angina, and investigation into plasma CK-MB concentration elevations in patients with unstable angina have yielded mixed results (Thygesen, K. et al., *Eur. J. Clin. Invest.* **16**:1-4, 1986; Koukkunen, H. et al., *Ann. Med.* **30**:488-496, 1998; Bertinchant, J.P. et al., *Clin. Biochem.* **29**:587-594, 1996; Benamer, H. et al., *Am. J. Cardiol.* **82**:845-850, 1998; Norregaard-Hansen, K. et al., *Eur. Heart J.* **13**:188-193, 1992). The mixed results associated with unstable angina suggest that CK-MB may be useful in determining the severity of unstable angina because the extent of myocardial ischemia is directly proportional to unstable angina severity. Elevations of the plasma CK-MB concentration are associated with skeletal muscle injury and renal disease. CK-MB is released into the bloodstream following cardiac cell death. The plasma concentration of CK-MB in patients with AMI is significantly elevated 4-6 hours after onset, peaks between 12-24 hours, and returns to baseline after 3 days. The release kinetics of CK-MB associated with unstable angina may be similar.

[0058] Glycogen phosphorylase (GP) is a 188 kDa intracellular allosteric enzyme that catalyzes the removal of glucose (liberated as glucose-1-phosphate) from the nonreducing ends of glycogen in the presence of inorganic phosphate during glycogenolysis. GP is present as a homodimer, which associates with another

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homodimer to form a tetrameric enzymatically active phosphorylase A. There are three isoforms of GP that can be immunologically distinguished. The BB isoform is found in brain and cardiac tissue, the MM isoform is found in skeletal muscle and cardiac tissue, and the LL isoform is predominantly found in liver (Mair, J. et al., *Br. Heart J.* **72**:125-127, 1994). GP-BB is normally associated with the sarcoplasmic reticulum glycogenolysis complex, and this association is dependent upon the metabolic state of the myocardium (Mair, J., *Clin. Chim. Acta* **272**:79-86, 1998). At the onset of hypoxia, glycogen is broken down, and GP-BB is converted from a bound form to a free cytoplasmic form (Krause, E.G. et al., *Mol. Cell Biochem.* **160-161**:289-295, 1996). The normal plasma GP-BB concentration is < 7 ng/ml (36 pM). The plasma GP-BB concentration is significantly elevated in patients with AMI and unstable angina with transient ST-T elevations, but not stable angina (Mair, J. et al., *Br. Heart J.* **72**:125-127, 1994; Mair, J., *Clin. Chim. Acta* **272**:79-86, 1998; Rabitzsch, G. et al., *Clin. Chem.* **41**:966-978, 1995; Rabitzsch, G. et al., *Lancet* **341**:1032-1033, 1993). Furthermore, GP-BB also can be used to detect perioperative AMI and myocardial ischemia in patients undergoing coronary artery bypass surgery (Rabitzsch, G. et al., *Biomed. Biochim. Acta* **46**:S584-S588, 1987; Mair, P. et al., *Eur. J. Clin. Chem. Clin. Biochem.*